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PROTEIN KINASE C FORMS A COMPLEX WITH AND PHOSPHORYLATES THE GTPase ACTIVATING PROTEIN GAP: PHOSPHORYLATION BY PKC IS DEPENDENT ON TYROSINE PHOSPHORYLATION OF GAP AND/OR A GAP-ASSOCIATED PROTEIN

Michael GSCHWENDT*, Walter KITTSTEIN and Friedrich MARKS

German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, Germany

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Protein kinase C (PKC) and the GTPase-activating protein GAP can be detected in immunoprecipitates of mouse epidermis and lung cytosol obtained with either anti-GAP or anti-PKC antisera. The PKC in the immune-complex phosphorylates the coprecipitated GAP protein. Moreover, purified recombinant GAP is phosphorylated in vitro by purified PKC. The efficacy of this phosphorylation appears to depend on the extent of tyrosine phosphorylation of GAP and/or a GAP-associated protein.

c-Ras is negatively regulated by a GTPase-activating protein (GAP; 1,2). Evidence was provided demonstrating the presence of a link between GAP and protein kinase C (PKC)-mediated signalling pathways. Downward et al. (3) showed that treatment of T cells with the phorbol ester TPA increased the relative amount of active GTP-bound ras in the cell membranes and that this ensued from a PKC-mediated reduction in GAP activity. According to Nori et al. (4), overexpression of GAP in NIH 3T3 cells blocks the TPA-induced activation of p42 mitogen-activated protein kinase, c-fos expression and DNA synthesis. Furthermore, GAP binds to and is phosphorylated by receptor tyrosine kinases (5-8). Taken together, this evidence strongly supports the concept that GAP plays an integral role in receptor tyrosine kinase and PKC-mediated signal transduction cascades.

Here we show by means of immunoprecipitation, using both PKC and GAP antibodies, that PKC and GAP from mouse epidermis and lung cytosol form a complex. PKC as part of this complex as well as purified PKC is able to phosphorylate GAP. PKC phosphorylation of GAP apparently is dependent on tyrosine phosphorylation of GAP and/or a GAP-associated protein.

MATERIALS AND METHODS

Materials

TPA and recombinant GAP were kindly provided by Drs. E. Hecker (German Cancer Research Center, Heidelberg, F.R.G.) and A. Wittinghofer (Max Planck Institute of Medical Research, Heidelberg, F.R.G.), respectively.

* To whom correspondence should be addressed.

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[γ-^{32}P]ATP (specific activity 5000 Ci/mmole) was from Du Pont-New England Nuclear.
Anti-PKCζ and anti-phosphotyrosine antibodies were from GIBCO BRL.
Anti-GAP antiserum and phosphotyrosine phosphatase-1B were from Upstate Biotechnology, Inc.
Protein A-agarose was from Boehringer.

Immunoprecipitation
500 μl of cytosol or of a particulate extract were incubated together with 10 μl of an antiserum for 2 h at 0-2 °C. After addition of 100 μl protein A-agarose, the mixture was incubated for another hour. The resulting immune complex was pelleted by centrifugation and washed three times with 400 mM NaCl, 50 mM Tris-HCl, pH 7.4. It was either used for phosphorylation (see below) or heated in SDS sample buffer and separated by SDS polyacrylamide gel electrophoresis.

Phosphorylation of proteins
Phosphorylation was performed in 120 μl of phosphorylation buffer [20 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 10 μg phosphatidyl serine (PS)]. Where indicated, 0.5 mM $CaCl_2$, 10^{-7} M TPA, and PKC were added). The reaction was started by addition of 5 μl of [γ-^{32}P]-ATP (a 1:4 mixture of 200 μCi ^{32}P-ATP (5000 Ci/mmol) and 750 μM ATP). After incubation at 30 °C for 10 min. proteins were either pelleted by addition of 10% trichloroacetic acid or pelleted by centrifugation and washed once with 500 μl of 400 mM NaCl, 50 mM Tris-HCl, pH 7.4 (phosphorylation in immunoprecipitates; see Fig.1). The protein pellets were then heated in SDS sample buffer and separated by electrophoresis on a 7.5% SDS polyacrylamide gel.

RESULTS AND DISCUSSION

We investigated the possibility of precipitation from the cytosol of murine epidermis and lung substrates of PKCζ together with the enzyme, using a PKCζ-specific anitserum. Both tissues are relatively rich in PKCζ, which is located predominantly in the cytosol (9). Incubation of the immunoprecipitates with [γ-^{32}P]-ATP, i.e. without addition of exogenous PKC, resulted in phosphorylation of a 120 kDa protein (see Fig.1). This protein was recognized by an antiserum directed against the GTPase-activating protein of ras (GAP; see Fig.2a). In order to prove the immunocoprecipitation of PKCζ and GAP, we performed the immunoprecipitation from epidermal cytosol with antisera against PKCζ or GAP (Fig.2a). Proteins in the immunoprecipitates were separated by gel electrophoresis and immunoblotted, again using GAP or PKCζ antisera. Both GAP and PKCζ immunoprecipitates contained 120 kDa GAP together with PKCζ, as recognized by the corresponding antisera (Fig.2a). Similar results were obtained with a PKCα antiserum, whereas with a control serum from non-immunized rabbits no precipitation of GAP and PKC was observed (data not shown). These findings indicate that GAP binds to different isoforms of PKC rather tightly and is phosphorylated by the kinase in the immune complex. The amount of GAP in epidermal cytosol was comparable to that in cytosol of brain (Fig.2b) as one of the tissues with the highest expression of GAP (10).

Phosphorylation of GAP by PKC was further investigated by means of purified proteins. As shown in Fig.3a, lane 3, GAP [expressed in and purified from insect cells (11)] was phosphorylated in the presence of Ca^{2+}, PS and phorbol ester TPA by purified cPKC (a mixture of PKCα,β,γ). Phosphorylation was neither observed in the absence of the enzyme (Fig.3a, lane 1) nor without cofactors (Fig.3a, lane 2). This indicated that the GAP preparation was free of PKC-related kinase activity and that phosphorylation of GAP entirely depended on the addition of PKC. In the autoradiogram, phosphorylated GAP appeared as a

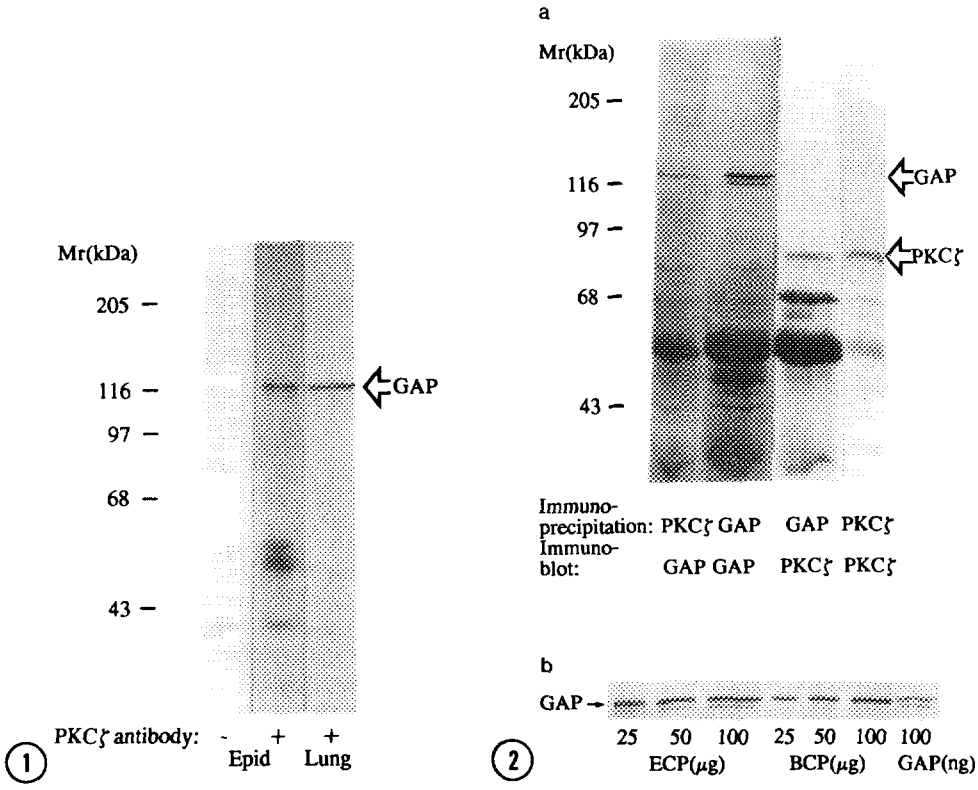

Fig. 1. Protein phosphorylation in the immunoprecipitates from murine epidermis and lung cytosol obtained with anti-PKCζ antiserum.
Epidermis of the back skin or lung of female NMRI mice was homogenized in 5 vol of 20 mM Tris-HCl, pH 7.4, 1 mM PMSF. The homogenates were separated into cytosol and particulate fraction by centrifugation at 100,000 x g for 30 min. 500 μl of cytosol (about 1.5 mg protein/ml) was immunoprecipitated with PKCζ antiserum and the proteins in the immunoprecipitate were phosphorylated in the absence of Ca^{2+}, TPA and exogenous PKC as described in Methods. Phosphorylated proteins were separated by electrophoresis on a 7.5% SDS polyacrylamide gel and visualized by autoradiography.

Fig. 2.a. Immunoprecipitation of PKCζ together with GAP from murine epidermis cytosol with anti-PKCζ or anti-GAP antiserum.
Immunoprecipitates obtained with anti-PKCζ antiserum or anti-GAP antiserum (see Methods) were separated by gel electrophoresis and immunoblotted (9,12,13), using PKCζ and GAP antisera as indicated.
b. Comparison of the content of GAP in murine epidermis (ECP) and brain cytosol (BCP). Various amounts of cytosol (μg protein) or 100 ng of recombinant full-length GAP purified from insect cells harboring a recombinant baculovirus (generous gift of Dr. A. Wittinghofer, Max Planck Institute of Medical Research, Heidelberg, F.R.G., see ref.11) were separated by gel electrophoresis, transferred to nitrocellulose and blotted with anti-GAP antiserum as above.

doublet around 120 kDa. A similar doublet was observed also in the immunoblots of epidermal cytosol (see Fig.1a). A second doublet with a somewhat lower molecular weight, which was also recognized by the GAP antibody (see Fig.3c) but was shown absent from epidermal cytosol, might represent proteolytic fragments of recombinant GAP caused by

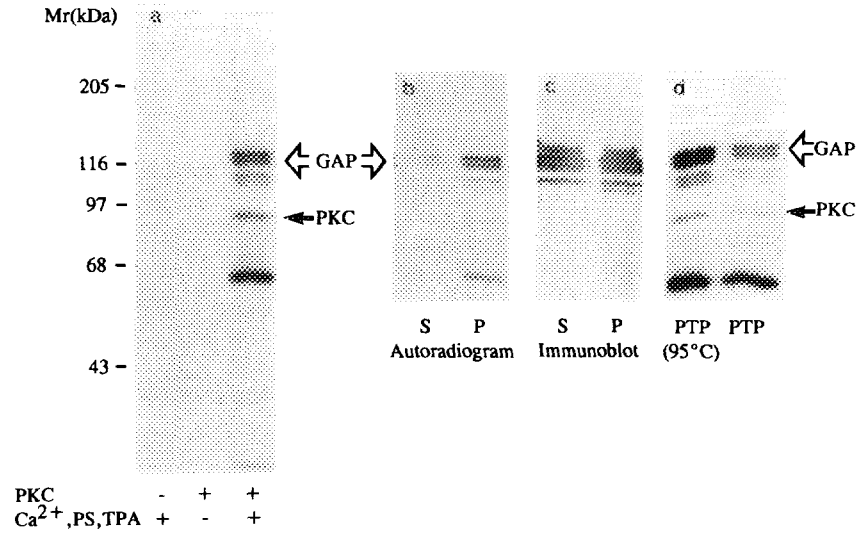

Fig. 3. a. Phosphorylation of purified recombinant GAP with cPKC.
1.3 μg of purified GAP (see Fig.2) in 60 μl of phosphorylation buffer was incubated with ^{32}P-ATP, in the presence or absence of purified cPKC (13) and Ca^{2+}, PS, TPA, as indicated (for details see Methods). The reaction was stopped by addition of 10% trichloroacetic acid, and the resulting precipitate was analyzed by gel electrophoresis and autoradiography.
b.,c. Immunoprecipitation of PKC-phosphorylated GAP with anti-phosphotyrosine antibodies. After phosphorylation of 2.6 μg of GAP with PKC (see above), 100 μl of 20 mM Tris-HCl, pH 7.4,and 10 μl (10 μg) of anti-phosphotyrosine antibodies were added. Immunoprecipitation was performed as described in Methods. The immunoprecipitate as well as the supernatant (after precipitation with 10% trichloroacetic acid) were separated by gel electrophoresis, transferred to nitrocellulose and either visualized by autoradiography (b) or immunoblotting with anti-GAP antiserum (c).
d. Phosphorylation of phosphotyrosine phosphatase-pretreated GAP with cPKC. 1.3 μg of purified GAP was incubated for 15 min. at 37 °C with 10 μl (0.5 μg) of either active phosphotyrosine phosphatase-1B (PTP) or inactivated PTP (95 °C, 10 min). Subsequently, GAP was phosphorylated with cPKC in the presence of Ca^{2+}, PS, TPA, and analyzed as described in Fig.3a.

partial degradation. Another protein with a molecular weight around 65 kDa was extensively phosphorylated by PKC and immunoprecipitated by an anti-phosphotyrosine antibody (see Fig.3b). The latter might be a GAP-associated protein similar to p62 that was copurified with GAP from the insect cells. GAP is known to associate with various proteins, such as p62 and p190 (7,14,15). Recently, we obtained preliminary results indicating that the GAP-associated protein p62 is phosphorylated by PKC.

PKC-phosphorylated GAP was almost completely immunoprecipitated by an anti-phosphotyrosine-antibody (see the autoradiogram, Fig.3b). In contrast, total GAP as recognized by immunoblotting with a GAP antibody was equally distributed between precipitate and supernatant (see the immunoblot, Fig.3c). This result indicates that only tyrosine-phosphorylated GAP was phosphorylated by PKC. Actually, only a small portion of GAP is phosphorylated at tyrosine (14). On the other hand, GAP could have been immunoprecipitated by the anti-phosphotyrosine antibody due to its association with another

tyrosine phosphorylated protein which might facilitate GAP-phosphorylation by PKC. Indeed, p62 contains a tyrosine-rich domain (15), and in src-transformed or EGF-stimulated cells it is even more intensively phosphorylated at tyrosine than GAP (7). Moreover, p62 appears to be required for translocation and binding of GAP to the plasma membrane (14), where phosphorylation by PKC is expected to occur. p65 found in our GAP preparation was also phosphorylated by PKC and the phosphorylated form could be completely precipitated by an anti-phosphotyrosine antibody (Fig.3b).

In order to further investigate a possible permissive role of tyrosine phosphorylation in PKC-catalyzed phosphorylation of GAP, we treated GAP with protein tyrosine phosphatase 1B (PTP) prior to phosphorylation by cPKC. As shown in Fig.3d, tyrosine dephosphorylation (right lane) caused a significant decrease in the incorporation of phosphate into GAP by cPKC, as compared to a control pretreated with inactivated PTP (left lane). PTP was unable to interfere with PKC-catalyzed phosphorylation of GAP or other PKC substrates, such as histone III-S (data not shown). This result supports our notion of a hierarchical phosphorylation of GAP, although an indirect effect, i.e. dephosphorylation and subsequent dissociation of a GAP-associated protein, cannot be ruled out at present.

Regulation of GAP activity appears to be a rather complex process. Although GAP is known to be phosphorylated at serine and tyrosine residues (7), neither the precise location of the phosphorylated amino acids (with the exception of tyr-460 of human GAP, which is phosphorylated by the EGF receptor, ref.16) nor the physiological role of each individual phosphorylation are known. GAP contains a potential PKC phosphorylation site (R R K T K) close to tyr-457 (tyr-457 of bovine GAP corresponds to tyr-460 of human GAP). The synthetic GAP peptide 452 to 468 containing this phosphorylation site is phosphorylated by cPKC as effectively as the cPKC pseudosubstrate (cPKC 19-31) with serine in position 25 (data not shown). However, phosphorylation of tyr-457 could not be shown as yet to affect the PKC phosphorylation of the GAP-peptide. Furthermore, GAP associates with other proteins, such as p62 or p190, which themselves are phosphorylated at serine and tyrosine (7). Phosphorylation of GAP and GAP-binding proteins might influence the formation of respective complexes and thus have marked effects on the GAP function. Complex formation and/or phosphorylation also appear to determine the intracellular localisation of GAP, e.g. in its association with plasma membranes (14). In summary, our results indicate that GAP binds to PKC and that GAP is a substrate of PKC. This interaction of GAP with PKC depends on a preceding tyrosine phosphorylation of GAP itself and/or an associated protein. Although tyrosine phosphorylation of GAP has been reported not to affect its GTPase-activity (16), its permissive role in other phosphorylations, and probably in the association with other proteins, might well affect the GAP function. Such a hierarchial phosphorylation of the GAP complex could be part of a cross-talk between tyrosine kinase and PKC-mediated signal transduction pathways.

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